Version Date: 7/26/2010

SCREEN FOR THE PRESENCE OF POLYCYCLIC AROMATIC HYDROCARBONS IN SELECT SEAFOODS USING LC-FLUORESCENCE

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Abstract

A liquid chromatography with fluorescence detection (LC-FLD) method has been developed to screen for fifteen targeted polycyclic aromatic hydrocarbons (PAHs) at concentrations below the established levels of concern in oysters, shrimp, crabs and finfish. The procedure was validated by spike recovery experiments at three levels for each matrix, and through analysis of NIST standard reference material SRM 1974b. PAHs are extracted using a modification of the quick, easy, cheap, effective, rugged and safe (QuEChERS) sample preparation procedure, employing acetonitrile (CH_3CN) as the solvent. The extracts are filtered using 0.2 micron syringe filters, but require no post-extraction sample cleanup for LC-FLD analysis. The chromatographic method employs a polymeric C18 stationary phase designed for PAH analysis by gradient elution to resolve fifteen targeted PAHs in a 35 minute run time. For the analysis of unknowns, a sample that is determined to be positive for a targeted PAH at or above 50% of the FDA level of concern requires that confirmatory analysis be performed. Additionally, an estimate of total PAH concentration including alkyl homologues in the sample is calculated. Samples containing total PAH concentrations greater than 50% of the FDA level of concern for naphthalene require that confirmatory analysis be performed.

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Introduction

Polycyclic aromatic hydrocarbons are found in crude oil in significant amounts, with some exceeding 2000 μg per kg (1). The EPA has reported that the metabolites of many of these compounds have been shown in laboratory test systems to be carcinogens, co-carcinogens, teratogens, and/or mutagens (2). As of July 20, 2010, the U.S. Department of Energy estimates that over 92,000,000 gallons of oil have spilled into the Gulf of Mexico in the recent Deepwater Horizon environmental tragedy (3). The sheer volume of this disaster in conjunction with the potential toxicity of the compounds involved is of particular concern to the commercial and recreational fishing industries. Previous environmental tragedies have prompted the development of methods to screen seafood entering the consumer market for the presence of PAHs. However, many previously accepted methods such as the NOAA Technical Memorandum NMFS-NWFSC-59 (4) require extensive clean up procedures as well as fraction collection using size exclusion chromatography. With the large amounts of domestic and exported seafood at risk, a simplified methodology allowing for high sample throughput is necessary.

Extensive research has been conducted by Krahn, *et al.* (5) in the use of liquid chromatography equipped with fluorescence detection for the analysis of petroleum related aromatic compounds. Two methods put forth by Ramalhosa, *et al.* (6) and Pule, *et al.* (7) make use of the AOAC QuEChERS (quick, easy, cheap, effective, rugged, and safe) method for sample preparation and employ the use of LC-FLD. This study has been adapted from the two previous methods and tested for applicability on a variety of seafood matrices including oysters, shrimp, fish, and crab. A variety of sample preparation procedures were explored and it was determined that the required sensitivity could be achieved using 5 g of sample, 15 mL of acetonitrile, and the $MgSO_4$/NaOAc step of the modified QuEChERS technique, with no additional sample cleanup.

Sensitivity of the method is one of the primary concerns. Benzo[a]pyrene, one of the most widely occurring and potent PAHs, as well as six other PAHs have been classified by the EPA as probable human carcinogens (2). The level of concern for benzo(a)pyrene has been established at 35 ng/g (8). This method's detection limit has been evaluated at a concentration of 5 ng/g, sufficiently low for the method to be used for screening purposes. Additionally, NIST standard reference material SRM 1974b (9) was used for further verification of the method.

This procedure is applicable to screen a variety of seafood matrices including oysters, shrimp, fish and crab for the presence of PAHs due to oil contamination. The objective of this work is to simplify existing methodology to increase throughput.

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Experimental

Equipment and Supplies

- Liquid chromatograph (Agilent 1200 series) with binary pump, microdegasser, autosampler, thermostatted column compartment and multiwavelength fluorescence detector.
- Agilent Chemstation software for controlling LC and data analysis
- Zorbax Eclipse PAH analytical column, rapid resolution HT, 4.6 x 50 mm 1.8 μm (Agilent p/n 959941-918)
- Zorbax Eclipse analytical guard column, 4.6 x 12.5 mm 5 μm (Agilent p/n 820950-939)
- Guard column hardware kit, high pressure (Agilent p/n 820888-901)
- Centrifuge capable of 3000 x g for 50 mL centrifuge tubes
- Balance, sensitivity of 0.1 mg
- Adjustable pipettes (10 - 100 μL, 100 - 1000 μL, and 1 - 10 mL) and tips
- Centrifuge tube racks for 50 mL (30 mm) tubes
- Robot Coupe processor with stainless steel bowl (Robot Coupe p/n R301UB)
- Buffered QuEChERS extraction tubes with foil packet containing 6 g of magnesium sulfate and 1.5 g of sodium acetate (AOAC Method 2007.01, for use with 15 g samples), (Agilent p/n 5982-5755)
- Ceramic homogenizers for 50 mL tubes (100/pk), (Agilent p/n 5982-9313)
- Syringes (without needles, nonsterile, BD Luer-Lok Tip, 5 mL capacity), (Fisher p/n 14-823-16D)
- PTFE syringe filters (0.20 μm pore size, 25 mm dia.), (Fisher p/n SLFG 025 NK) *Note: to minimize interferences, it is critical that PTFE not be substituted with nylon or other materials.*
- 4 mL amber glass vials with PTFE lined caps, (Fisher p/n B7800-2A)

All equipment and supplies listed may be substituted with equivalent.

Reagents and Standards

- Acetonitrile, HPLC grade (Fisher p/n A998, or equivalent)
- Water, 18.2 MΩ water from a Millipore Milli-Q Gradient A-10 water source (or equivalent) referred to as RODI (reverse osmosis de-ionized)
- QTM PAH Mix (contains 16 PAH @ 2000 micrograms/mL each in methylene chloride) (Supelco p/n 47930-U, or equivalent)
- Benzo(k)fluoranthene, (Supelco p/n 48492, or equivalent)
- NIST Standard Reference Material 1974b, Organics in Mussel Tissue (*Mytilus edulis*)

Stock Standards Preparation

A stock standard solution of benzo(k)fluoranthene was prepared at a concentration of 2000 µg/mL in methylene chloride. The QTM PAH Mix is a solution that contains a mixture of sixteen PAHs in methylene chloride, each at a concentration of 2000 µg/mL.

250 µg/mL stock standard: 10 mL of a stock spiking solution was prepared by combining 1.25 mL each of the QTM PAH mix and the 2000 µg/mL benzo(k)fluoranthene stock, followed by dilution to 10 mL with CH_3CN

5.0 µg/mL stock standard: 25 mL of a 5 µg/mL spiking solution was prepared by adding 500 microliters of the 250 µg/mL stock standard to a 25 mL volumetric flask and diluting to volume with CH_3CN

0.5 µg/mL stock standard: 25 mL of a 0.5 µg/mL spiking solution was prepared by adding 2.5 mL of the 5 µg/mL stock standard to a 25 mL volumetric flask and diluting to volume with CH_3CN

Calibration Standards

Calibrations standards were prepared at concentrations of 2.5, 25, and 50 ng/mL to demonstrate linearity. Dilutions (1:10 and 1:20) of the 0.5 µg/mL stock standard with CH_3CN were used to prepare the 50, and 25 ng/mL calibration standards; and a further 1:10 dilution of the 25 ng/mL calibration standard was used to prepare the 2.5 ng/mL calibration standard.

Check Standards/CCV Standards

For validation studies of laboratory fortified matrices, the check standard is an external standard made to the same final concentration as the spiked matrix samples. Check standards were used for calculation of all sample spike/recoveries based on the peak area ratios of the spiked matrix sample to the appropriate check standard. The check standard is prepared by serial dilution of the nominal 250 µg/mL stock standard spiking solution. Refer to Table 1 for preparation of check standards. All dilutions are prepared in acetonitrile. *Equivalent dilution schemes may be substituted.*

For sample analysis, a continuing calibration verification (CCV) standard is analyzed at the beginning and end of each batch of 20 or fewer samples. Typically, this standard is at a concentration near the middle of the calibration range such as the 16.7 ng/mL standard.

Table 1. Dilution scheme for preparation of check standards used in validation studies

Spiking Level	Spiked Matrix Sample Solution Final Concentration(ng/mL)	Check Standard Preparation
High and Mid	33.3	First Dilution: Prepare a 1000-fold dilution of the 250 µg/mL stock standard spiking solution by taking a 100 µl aliquot into a 100 ml volumetric flask, and diluting to volume.
		Second Dilution: Prepare a 7.5-fold dilution of the first dilution by taking 133 µl first dilution plus 867 µl CH_3CN. Use the second dilution as the check standard for the high and mid level spiked samples.
Low (oysters, crab, shrimp)	16.7	Third Dilution (oysters, crab, shrimp): Prepare a 2-fold dilution of the second dilution by taking 500 µl second dilution plus 500 µl CH_3CN. Use the third dilution as the check standard for the low level spiked samples of oysters, crab, and shrimp.
Low (finfish)	8.31	Third Dilution (finfish): Prepare a 4-fold dilution of the second dilution by taking 250 µl second dilution plus 750 µl CH_3CN. Use the third dilution as the check standard for the low level spiked samples of finfish.

Sample Composite Preparation

Seafood samples should be prepared by first obtaining the edible portion as described in Table 2. Samples are then composited and homogenized by blending in Robot Coupe food processor or equivalent for 2-3 minutes. Seafood samples were stored frozen, but partially thawed prior to preparation for analysis. The matrices used in this validation study included uncooked shrimp purchased at a local grocery store; oysters harvested from Louisiana; and finfish (Spanish Mackerel) and crab harvested from Alabama. Compositing of multiple individuals from the same site may be appropriate. The minimum sample size for this analysis is 5 g

Extraction Procedure

Finfish, Shrimp and Crab

For analysis of finfish, shrimp and crab, 5 grams of homogenized sample composite and a ceramic homogenizer are transferred to a QuEChERS extraction tube. Five grams of RODI water are then added to the extraction tube followed by vortex mixing or shaking for 1 minute. A 15 mL volume of CH_3CN is added to the extraction tube followed by a second one

minute vortex or shaking step. Next, the contents of the QuEChERS extraction foil packet (6 g of magnesium sulfate and 1.5 g of sodium acetate) are added to the mixture. The mixture is shaken vigorously for 1 minute; and the extract is centrifuged at 3000 x g for 10 minutes, allowing for removal of the CH_3CN (upper) layer (approx 6-8 mL). A portion (approximately 4 mL) of the supernatant extract is filtered through a 0.2 μm PTFE syringe filter into an amber glass vial and analyzed without further dilution using LC-FLD.

Table 2. Directions for obtaining edible tissue portion of selected matrices

Finfish	Remove heads, tails, scales, fins, viscera and bones, save edible portion. If the skin is considered edible, collect it as well.
Crab	Remove the front claw (propus) and the next section of the claw (merus), break the pincher off by pulling down on it. Insert an oyster tool into the opening and break the exoskeleton so that the meat inside can be removed. With the crab head up pull off the top shell (carapace) and discard. Remove viscera and gills. Collect the meat that is around the outer edge of the bottom section of the crab. These are in cartilage sections; an oyster tool can be used to break through the cartilage to obtain the small portion of meat. Approximately 20 grams of meat was typically obtained from a 6 inch blue crab.
Shrimp	Remove the head, shell, legs, and tail. Save the remaining edible portion.
Oyster	Find an opening between the top and bottom shell of the oyster to wedge the oyster tool into. When the correct location is found, a small amount of liquid inside the oyster will seep out around the edge where the 2 halves of the oyster come together. Pry the 2 shells apart, then scrape and collect all of the insides including the liquor.

Oysters

For analysis of oysters, the extraction differs only in that no water is added to the sample, thereby eliminating one mixing step described above for the other matrices. The addition of water to homogenized oyster samples was determined to be unnecessary due to the amount of water present in the native tissue.

SRM 1974b Organics in Mussel Tissue

The analysis of SRM 1974b in triplicate is required as an initial demonstration of accuracy. For SRM 1974b, the extraction procedure is identical to that described for oysters. Due to the low levels of PAHs in the SRM, a ten fold concentration step of the filtered extract is necessary. This is accomplished by evaporating 1 mL of extract to dryness under a stream of dry air without heating followed by reconstitution with 100 μL of acetonitrile.

*Fortification / Spike Recovery Sample*s

The analysis of one fortified sample matrix with each batch of 20 or fewer samples is required. Five grams of homogenized composite is fortified with 50 µL of the 5.0 µg/mL spiking solution. This provides a fortification level of 0.05 µg/g of sample.

Method Blanks

A method blank must be analyzed with each batch of 20 or fewer samples to monitor for contamination from laboratory sources. Additionally, a solvent blank should be analyzed between one or more samples to demonstrate lack of carry over from run to run.

Method blanks and fortified method blanks are prepared by substituting 5g of RODI water in place of sample composite and performing the extraction procedure as for oysters described above.

Liquid Chromatography with FLD Analysis

Samples, standards and blanks were analyzed using an Agilent 1200 Series liquid chromatograph equipped with a binary pump, microdegasser, autosampler, thermostatted column compartment and a fluorescence detector, all operated under the control of Chemstation software.

Separation of PAHs was accomplished at a flow rate of 0.8 mL/min on a Zorbax Eclipse PAH Rapid Resolution HT (4.6 x 50 mm, 1.8 µm) column with a Zorbax Eclipse Analytical Guard Column (4.6 x 12.5 mm, 5 µm). The mobile phase consisted of water and acetonitrile run as a gradient with conditions described in Table 3. The column thermostat was set to 18 ^{o}C and all injections were 10 µL.

Table 3. Gradient Program

time	volume % acetonitrile	volume % water	Comment
0	60	40	Analysis (from 0 to 30 min.)
1.5	60	40	
7.0	90	10	
13.0	100	0	
30.00	100	0	
30.01	60	40	Re-equilibration to initial conditions (from 30 – 35 min.)
35.00	60	40	

For fluorescence detection, an excitation wavelength of 260 nm was used for all 15 PAHs. However, programmable wavelength switching was used to optimize emission response, thereby improving sensitivity for individual PAHs and minimizing interferences. In addition, the PMT gain was adjusted to increase sensitivity. The detection signal timetable provided in Table 4 gives the excitation and emission wavelengths in addition to the photomultiplier gain settings used. Multiple emission wavelengths may be monitored (rather than wavelength switching) as long as sufficient sensitivity is maintained.

Table 4. Fluorescence Detection Signal Timetable

Time (min.)	Excitation nm	Emission nm	PMT-Gain	Baseline	PAHs detected
0.00	260	352	13	Zero	naphthalene, acenaphthene, fluorine, phenanthrene
6.35	260	420	13	Zero	anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[ghi]perylene
17.15	260	460	13	Zero	indeno[1,2,3-cd]pyrene

Data Analysis

Peak Identification

Individual chromatographic peaks were identified based on comparison of their retention times to those of known reference standards. Variability of the LC/FLD retention times should be within 1% of the corresponding standard for peak identification in samples.

Quantitation of Individual PAHs

Concentrations of individual PAHs are determined by comparison of sample peak areas to the peak areas of reference standards at known concentrations prepared in acetonitrile (external calibration). Calculations may be based on generated external calibration curves or CCV standards.

Concentrations of individual PAH concentrations (ng/g) in the samples are calculated as follows:

$$\text{PAH in sample (ng/g)} = \text{PAH in extract (ng/mL)} \times \frac{15 \text{ mL } CH_3CN}{5 \text{ g sample}}$$

When an analyte is not detected in a sample or it has a response area that is below the LOD, report less than the LOD. When the analyte response is greater than the LOD and less than the LOQ, the result should be reported as trace.

Estimation of Total PAH Concentration

A conservative estimate of the total amount of PAHs in samples including alkyl homologs is determined using the total area determined over the RT range of 2.5 – 20 minutes and the sensitivity (slope of the calibration curve) for the least sensitive parent compound from the following list: naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene.

Example (using slope date in Table 6):

A sample is analyzed and found to contain 10 peaks in the RT range of 2.5 – 20 minutes. Peaks may or may not match RT for known parent compounds.

$$\text{Total PAHs in extract (ng/mL)} = \frac{\text{Area found for integration 2.5 -20 min}}{\text{Slope for Naphthalene (0.512)}}$$

$$\text{Total PAHs in sample (ng/g)} = \text{Total PAHs in extract} \times \frac{15 \text{ mL } CH_3CN}{5 \text{ g sample}}$$

Criteria for Confirmatory Analysis

The LC-FLD method described in this document is considered to be a screening method for PAH contamination in seafood. Any positive or indeterminate findings must be confirmed using the NOAA method (4). Sample results from the LC-FLD method shall be evaluated for 1) individual parent PAH concentrations and 2) estimated total PAH concentration.

Individual parent PAH concentrations.
The parent PAH compounds for which levels of concern have been established are listed in Table 5. For the LC-FLD screening method, the levels requiring confirmatory analysis have been set at 50% of the FDA established levels of concern. If any one or more parent PAH concentrations exceeds these levels, the sample must undergo confirmatory analysis.

Estimated total PAH concentration.
The estimated total PAH concentration in the sample must not exceed 50% of the FDA established level of concern for naphthalene as shown in Figure 5. Thus for shrimp and crab the estimated total PAH level requiring confirmatory analysis is 61.5 mg/kg. While in oysters and finfish, the estimated total PAH levels requiring confirmatory analysis are 66.5 and 16.3 mg/kg, respectively.

QC Elements

- A minimum of three calibration standards must be analyzed to demonstrate linearity with $r^2 \geq 0.99$ for all analytes.

- The analysis of SRM 1974b in triplicate is required as an initial demonstration of accuracy and precision. Analysis of SRM 1974b should fall within the acceptable range (see Table 11) for 8 or more of the PAHs screened.

- The Limit of Detection (LOD) for a given analyte should be determined according to 40 CFR Part 136[5] using a minimum of 5 replicates of matrix recoveries fortified with approximately 5 µg/kg for each of the PAHs identified in Table 5. The following equation should be used:

  $$LOD = s \times t_{(n-1,\ 1-\alpha=0.99)}$$

  Where s = the standard deviation of the result and $t_{(n-1,\ 1-\alpha=0.99)}$ = students' t-value appropriate for a 99% confidence level and (n-1) degrees of freedom

- The Limit of Quantitation (LOQ) for a given analyte should be determined according to 40 CFR Part 136[5] using a minimum of 5 replicates of matrix recoveries fortified with approximately 5 µg/kg for each of the PAHs identified in Table 5. The following equation should be used:

  $$LOQ = 10 \times s$$

  Where s = the standard deviation of the result

- Continuing calibration verification (CCV) standards should be analyzed at the beginning and end of each batch of 20 or fewer samples. If CCV results do not meet specified criteria, then the entire batch and calibration standards must be reanalyzed. The CCV standards' RSD of the PAH responses relative to the internal standard must be $\leq$ 15 percent for the replicates.

- A minimum of one fortified sample matrix with each batch of 20 or fewer samples is required. Recoveries of the 0.05 µg/g PAH spike through the method must be in the range 60% - 130%. The retention times in the spiked samples should be within 1% of the RT of the corresponding standard.

- A minimum of one method blank made with 5 g RODI water in place of sample matrix must be analyzed with each batch of 20 or fewer samples. The PAH concentrations found in the method blank should be subtracted from the concentrations found in the samples. Some PAHs, such as naphthalene, are ubiquitous and may be difficult to eliminate. With the exception of benzo(a)pyrene, higher background levels may be acceptable. Concentrations in the method blank should not exceed 3 times the certified concentration for the PAH in SRM 1974b.

- A minimum of one sample replicate must be analyzed with each batch of 20 or fewer samples. For triplicate replicates, the precision is considered acceptable if the percent relative standard deviation (RSD) is $\leq$ 15 percent for all analytes. For duplicate replicates, this translates to a relative percent difference of $\leq$ 30 percent for all analytes.

Table 5. FDA established levels of concern for PAHs in shrimp, crab, finfish and oysters along with LC-FLD screen levels requiring confirmation analysis.

Compound	*FDA level of concern (mg/kg)*			*LC-FLD screen levels requiring confirmation analysis (mg/kg)*		
	Shrimp and Crab	**Oysters**	**Finfish**	**Shrimp and Crab**	**Oysters**	**Finfish**
Naphthalene	123	133	32.7	61.5	66.5	16.3
Acenapthene	NA	NA	NA	NA	NA	NA
Fluorine	246	267	65.3	123	133	32.6
Phenanthrene*				923	1000	245
Anthracene*	1846[1]	2000[1]	490[1]	923	1000	245
Fluoranthene	246	267	65.3	123	133	32.6
Pyrene	185	200	49.0	92.5	100	24.5
benz(a)anthracene	1.32	1.43	0.35	0.66	0.71	0.17
Chrysene	132	143	35.0	66	71.5	17
benzo(b)fluoranthene	1.32	1.43	0.35	0.66	0.71	0.17
benzo(k)fluoranthene	13.2	14.3	3.5	6.6	7.1	1.7
benzo(a)pyrene	0.132	0.143	0.035	0.066	0.071	0.017
dibenz(a,h)anthracene	0.132	0.143	0.035	0.066	0.071	0.017
benzo[ghi]perylene*	NA	NA	NA	NA	NA	NA
indeno[1,2,3-cd]pyrene	1.32	1.43	0.35	0.66	0.71	0.17

NA = not applicable

[1] *Represents the sum of level of concern for phenanthrene and anthracene*

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Results and Discussion

Sample Cleanup

Dispersive solid phase extraction (SPE) cleanup has been used in combination with QuEChERS extraction for PAH analysis (7). In this work, the use of primary secondary amine (PSA) and PSA in combination with C18 SPE was evaluated in preliminary spike recovery studies. It was determined that the additional cleanup offered little to no advantage and was therefore eliminated from the procedure. As a result, this method requires no post-extraction sample cleanup.

Chromatography

The conditions for the chromatographic separation of PAHs were taken directly from previous work (7) and required no further optimization other than adjustments made to the PMT gain setting. A representative chromatogram obtained for a standard mixture of the 15 PAHs at concentrations of 33 ng/mL each is presented in Figure 1. Good separation was achieved considering the structural similarities of many of these compounds. The retention window for these compounds is 3.1 to 17.5 minutes.

Three point calibration curves were generated for each of the selected PAHs at concentrations of 2.5, 25 and 50 ng/mL prepared in acetonitrile. Table 6 summarizes the calibration data for each PAH. The calibration standards were prepared and analyzed in triplicate and were linear in this range for each compound with correlation coefficients ranging from 0.99986 to 1.00000. The relative standard deviation (RSD) obtained for the retention time was less than 0.5% for all of the PAH calibration standards.

Instrument detection limits (IDL) and limits of quantitation (LOQ) are also presented in Table 6. They were determined by replicate analyses of a 1. 7 ng/mL standard mixture (n=7). IDL and LOQ values were determined as outlined below using the Student's t-test at a 99% confidence interval.

IDL = $t_{\alpha=99} \times s$, where s is the standard deviation and, for n=7, n-1=6, $t_{\alpha=99}$ = 3.7

IDL = 3.7 s

LOQ = 10 s

The average IDL for the 15 PAHs was 0.26 ng/mL with an average LOQ of 0.71 ng/mL. However, the average is somewhat skewed due to the high limits determined for indeno[1,2,3-cd]pyrene. Average IDLs and LOQs calculated when indeno[1,2,3-cd]pyrene is excluded drop to 0.10 ng/mL and 0.27, respectively.

Table 6. Calibration Summary

Compound	Calibration Range (n = 3) ng/mL	Retention Time (n = 9) Min	Retention Time (n = 9) %RSD	Regression equation[1] (n = 3)	R^2	IDL (n = 7) (ng/mL)	LOQ (n = 7) (ng/mL)
Naphthalene	0.0 - 50.0	3.1	0.49	y = 0.512x + 0.034	0.99996	0.17	0.46
Acenapthene	0.0 - 50.0	4.9	0.27	y = 0.689x + 0.089	0.99986	0.12	0.33
Fluorine	0.0 - 50.0	5.1	0.25	y = 1.869x + 0.033	0.99999	0.05	0.15
Phenanthrene	0.0 - 50.0	5.9	0.18	y = 3.433x + 0.174	0.99998	0.04	0.11
Anthracene	0.0 - 50.0	6.6	0.13	y = 8.454x + 0.088	1.00000	0.03	0.07
Fluoranthene	0.0 - 50.0	7.3	0.10	y = 0.517x + 0.025	0.99999	0.32	0.85
Pyrene	0.0 - 50.0	7.9	0.08	y = 0.936x + 0.023	0.99998	0.09	0.24
benz[a]anthracene	0.0 - 50.0	9.5	0.05	y = 3.589x - 0.025	1.00000	0.04	0.11
Chrysene	0.0 - 50.0	10.1	0.06	y = 1.409x + 0.137	0.99993	0.12	0.33
benzo[b]fluoranthene	0.0 - 50.0	11.8	0.06	y = 3.186x - 0.132	1.00000	0.05	0.14
benzo[k]fluoranthene	0.0 - 50.0	12.9	0.06	y = 14.209x - 0.272	1.00000	0.01	0.03
benz[a]pyrene	0.0 - 50.0	13.7	0.06	y = 7.646x + 0.257	0.99999	0.06	0.16
dibenz[a,h]anthracene	0.0 - 50.0	15.4	0.07	y = 0.671x - 0.072	0.99997	0.16	0.42
benzo[g,h,i]perylene	0.0 - 50.0	16.2	0.07	y = 1.374x - 0.169	0.99997	0.12	0.32
indeno[1,2,3-cd]pyrene	0.0 - 50.0	17.4	0.07	y = 0.268x - 0.204	0.99923	2.5	6.8

[1] y = area; x = concentration (ng/mL)

Determination of PAH content in Select Seafoods

The method was applied to the analysis of edible portions of oysters, finfish, shrimp and crabs. Validation of the method was accomplished, in part, by evaluating spike recoveries for each matrix, fortified in triplicate with 3 concentrations of 15 PAHs. Method detection limits (MDL) and limits of quantitation (LOQ) for 15 PAHs were determined for each matrix using a low level sample fortification.

For the method validation studies, 5 g portions of homogenized composite of each matrix type were fortified with fifteen selected PAHs at three different concentrations (low, mid and

high). The high fortification level in each matrix was 10 μg/g and was accomplished by the addition of 200 μL of a 250 μg/mL spiking solution. The mid level spike was prepared at 1.0 μg/g by the addition of 20 μL of a 250 μg/mL spiking solution. The low level spike for shrimp, crabs and oysters was prepared at 0.05 μg/g and was accomplished by the addition of 50 μL of a 5.0 μg/mL spiking solution. The low spike level for finfish was 0.025 μg/g and was accomplished by the addition of 25 μL of a 5.0 μg/mL spiking solution.

For the mid and high spike levels, an additional dilution of the extract was required to keep from saturating the detector. For the mid level spikes a 1:10 dilution with CH_3CN was performed just prior to analysis. For the high level spikes a 1:100 dilution with CH_3CN was performed just prior to analysis.

Additionally, five replicates were fortified at 5 ng/g for each matrix and each PAH. MDL and LOQ values were determined as outlined below using the Student's t-test at a 99% confidence interval.

MDL = $t_{\alpha=99} \times s$, where s is the standard deviation and, for n=5, n-1=4, $t_{\alpha=99} = 4.6$

MDL = 4.6 s

LOQ = 10 s

Analysis of Oysters

Representative chromatograms of oyster tissue, unfortified and fortified with 15 PAHs at a level of 1.0 μg/g, is presented in Figure 2. Figures of merit derived from these experiments are provided in Table 7. Average spike recoveries ranged from 76% to 101%. The RSD values for retention times of the 15 PAHs in oysters were all less than 0.5%. The method detection limits ranged from 0.39 ng/g for benzo[b]fluoranthene to 7.3 ng/g for indeno[1,2,3-cd]pyrene. The average MDL for all PAHs evaluated was 1.6 ng/g with an average method LOQ of 3.5 ng/g.

Table 7. PAH recoveries from homogenized oyster samples at three fortification levels.

	Average Retention Time (n = 14)		Oyster Spike Recovery Data (n=3)						MDL (n = 5)	LOQ (n = 5)
			Spike Level 10.0 µg/g		Spike Level 1.00 µg/g		Spike Level 50.0 ng/g			
Compound	**min**	**% RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	ng/g	ng/g
Naphthalene	3.096	0.44%	89	2.0%	86	2.9%	101	1.3%	3.5	7.5
Acenapthene	4.868	0.36%	90	2.7%	86	1.8%	91	1.5%	2.2	4.8
Fluorene	5.126	0.35%	90	2.1%	87	3.3%	91	2.0%	0.74	1.6
Phenanthrene	5.862	0.28%	89	2.0%	85	3.0%	93	0.4%	0.81	1.8
Anthracene	6.653	0.22%	88	2.2%	84	2.8%	91	0.8%	1.2	2.6
Fluoranthene	7.343	0.18%	90	1.4%	83	3.1%	76	0.3%	0.89	1.9
Pyrene	7.883	0.26%	89	1.1%	83	3.1%	87	1.9%	0.80	1.7
benz[a]anthracene	9.562	0.11%	90	1.1%	86	2.9%	96	1.3%	0.71	1.5
Chrysene	10.120	0.06%	90	0.4%	87	2.9%	95	2.0%	1.3	2.9
Benzo[b]fluoranthene	11.832	0.08%	89	1.5%	83	2.6%	88	0.7%	0.39	0.84
Benzo[k]fluoranthene	12.949	0.08%	88	1.8%	83	2.9%	93	0.6%	0.54	1.2
Benzo[a]pyrene	13.739	0.07%	88	1.5%	83	2.9%	87	0.8%	0.47	1.0
Dibenz[a,h]anthracene	15.439	0.09%	87	1.2%	81	3.1%	88	0.4%	1.7	3.8
Benzo[g,h,i]perylene	16.213	0.09%	85	1.5%	78	3.8%	87	1.7%	1.7	3.6
Indeno[1,2,3-cd]pyrene	17.469	0.10%	87	2.0%	80	3.6%	98	3.3%	7.3	16

Analysis of Finfish

Representative chromatograms of finfish (Spanish Mackerel) edible tissue, unfortified and fortified with 15 PAHs at a level of 25 ng/g, is presented in Figure 3. Figures of merit derived from these experiments are provided in Table 8. Average spike recoveries ranged from 69% to 112%. The RSD values for retention times of the 15 PAHs in finfish were all less than 0.65%. The method detection limits ranged from 0.11 ng/g for benz[a]anthracene to 2.2 ng/g for indeno[1,2,3-cd]pyrene. The average MDL for all PAHs evaluated in finfish was 0.61 ng/g with an average method LOQ of 1.3 ng/g.

Table 8. PAH recoveries from homogenized finfish samples at three fortification levels.

	Average Retention Time (n = 14)		Finfish Spike Recovery Data (n=3)						MDL (n = 5)	LOQ (n = 5)
			Spike Level 10.0 µg/g		Spike Level 1.00 µg/g		Spike Level 25.0 ng/g			
Compound	**min**	**% RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**(ng/g)**	**(ng/g)**
Naphthalene	3.084	0.61%	84	2.7%	83	5.7%	112	0.6%	0.46	1.0
Acenapthene	4.862	0.25%	88	1.4%	86	4.5%	81	2.4%	0.85	1.8
Fluorene	5.119	0.24%	89	2.2%	88	5.0%	86	1.9%	0.27	0.58
Phenanthrene	5.855	0.21%	88	1.8%	86	4.9%	107	1.4%	1.3	2.8
Anthracene	6.644	0.18%	85	3.2%	84	5.0%	84	2.2%	0.12	0.27
Fluoranthene	7.333	0.16%	88	2.8%	85	3.8%	85	1.1%	1.8	4.0
Pyrene	7.865	0.14%	84	2.9%	83	5.2%	82	1.9%	0.69	1.5
benz[a]anthracene	9.548	0.13%	85	1.3%	84	5.2%	83	2.2%	0.11	0.24
Chrysene	10.110	0.15%	86	1.4%	85	5.4%	81	2.3%	0.31	0.66
Benzo[b]fluoranthene	11.811	0.15%	85	1.6%	83	5.1%	83	1.8%	0.18	0.39
Benzo[k]fluoranthene	12.916	0.18%	86	1.7%	83	4.9%	86	1.9%	0.15	0.34
Benzo[a]pyrene	13.707	0.17%	79	2.7%	78	5.4%	77	4.5%	0.16	0.36
Dibenz[a,h]anthracene	15.393	0.17%	83	1.7%	81	5.0%	80	4.2%	0.29	0.63
benzo[g,h,i]perylene	16.167	0.16%	77	1.8%	75	5.4%	75	3.4%	0.24	0.52
Indeno[1,2,3-cd]pyrene	17.399	0.21%	82	12%	74	15%	69	1.7%	2.2	4.7

Analysis of Shrimp

Shrimp edible tissue was also fortified at three concentration levels and the results are presented in Table 9. Figures of merit derived from these experiments are provided in Table 9. Average spike recoveries ranged from 76% to 116%. The RSD values for retention times of the 15 PAHs in shrimp were all less than 0.15%. The method detection limits ranged from 0.23 ng/g for benz[a]anthracene to 8.2 ng/g for indeno[1,2,3-cd]pyrene. The average MDL for all PAHs evaluated in shrimp was 2.6 ng/g with an average method LOQ of 5.7 ng/g.

Table 9. PAH recoveries from homogenized shrimp samples at three fortification levels.

	Average Retention Time (n = 14)		Shrimp Spike Recovery Data (n=3)						MDL (n = 5)	LOQ (n = 5)
			Spike Level 10.0 µg/g		Spike Level 1.00 µg/g		Spike Level 50.0 ng/g			
Compound	**min**	**% RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**ng/g**	**ng/g**
Naphthalene	3.096	0.12%	90	1.8%	86	0.54%	116	4.1%	6.9	15
Acenapthene	4.858	0.08%	91	2.0%	87	0.55%	91	0.95%	10	22
Fluorene	5.115	0.09%	93	1.6%	90	0.58%	93	1.1%	1.4	3.1
Phenanthrene	5.852	0.07%	92	1.3%	89	0.62%	89	1.5%	0.91	2.0
Anthracene	6.643	0.07%	92	1.3%	89	0.88%	90	1.3%	0.18	0.39
Fluoranthene	7.332	0.06%	94	1.2%	90	0.81%	82	4.9%	5.9	13
Pyrene	7.864	0.06%	92	1.5%	89	0.07%	86	2.7%	0.93	2.0
benz[a]anthracene	9.550	0.06%	92	1.1%	88	1.1%	89	1.3%	0.53	1.1
Chrysene	10.113	0.07%	93	0.86%	89	2.0%	87	2.3%	0.95	2.1
Benzo[b]fluoranthene	11.815	0.09%	91	1.1%	87	1.8%	88	1.2%	0.23	0.50
Benzo[k]fluoranthene	12.928	0.10%	92	1.1%	88	1.6%	92	1.4%	0.24	0.52
Benzo[a]pyrene	13.718	0.10%	90	1.2%	87	1.6%	87	1.9%	0.39	0.86
Dibenz[a,h]anthracene	15.405	0.11%	89	0.94%	85	1.5%	88	1.7%	1.7	3.7
Benzo[g,h,i] perylene	16.176	0.10%	86	1.5%	82	2.0%	82	2.5%	0.84	1.8
Indeno[1,2,3-cd] pyrene	17.416	0.12%	87	6.1%	77	5.1%	76	4.7%	8.2	18

Analysis of Crabs

Crab edible tissue was also fortified at three concentration levels and the results are presented in Table 10. Figures of merit derived from these experiments are provided in Table 10. Average spike recoveries ranged from 83% to 116%. The RSD values for retention times of the 15 PAHs in crab were all less than 0.35%. The method detection limits ranged from 0.33 ng/g for benzo[k]fluoranthene to 20 ng/g for indeno[1,2,3-cd]pyrene. The average MDL for all PAHs evaluated in crab was 2.9 ng/g with an average method LOQ of 6.3 ng/g.

Table 10. PAH recoveries from homogenized crab samples at three fortification levels.

	Average Retention Time (n = 14)		Crab Spike Recovery Data (n=3)						MDL (n = 5)	LOQ (n = 5)
			Spike Level 10.0 µg/g		Spike Level 1.00 µg/g		Spike Level 50.0 ng/g			
Compound	**min**	**% RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**% recovery**	**%RSD**	**ng/g**	**ng/g**
Naphthalene	3.099	0.31%	85	3.7%	87	7.4%	116	2.5%	8.7	19
Acenapthene	4.861	0.14%	86	4.9%	90	7.5%	85	4.8%	2.0	4.3
Fluorine	5.118	0.12%	86	4.3%	89	3.3%	89	1.8%	0.51	1.1
phenanthrene	5.855	0.09%	88	4.3%	91	2.9%	90	2.2%	0.90	2.0
Anthracene	6.645	0.08%	89	4.0%	91	2.2%	87	1.3%	0.55	1.2
Fluoranthene	7.334	0.06%	91	4.8%	92	2.6%	87	0.60%	1.5	3.3
Pyrene	7.866	0.06%	91	4.2%	92	1.8%	89	2.1%	1.1	2.4
benz[a]anthracene	9.552	0.06%	92	3.4%	92	1.7%	89	2.0%	0.76	1.7
Chrysene	10.115	0.06%	94	2.3%	92	0.38%	87	2.3%	0.58	1.3
benzo[b]fluoranthene	11.818	0.08%	92	3.9%	92	2.1%	88	1.5%	0.56	1.2
Benzo[k]fluoranthene	12.933	0.10%	93	3.3%	91	1.9%	91	1.6%	0.33	0.71
Benzo[a]pyrene	13.723	0.10%	93	3.4%	91	2.1%	87	2.1%	0.49	1.1
Dibenz[a,h]anthracene	15.413	0.12%	94	2.9%	94	2.1%	90	1.7%	3.4	7.3
benzo[g,h,i] perylene	16.183	0.12%	91	2.8%	91	0.99%	89	2.7%	2.1	4.6
Indeno[1,2,3-cd] pyrene	17.426	0.19%	92	8.9%	101	3.9%	83	11%	20	44

Analysis of NIST SRM 1974b Organics in Mussel Tissue

One component of the validation of the optimized method was accomplished by analyzing NIST SRM 1974b (9), a frozen mussel tissue homogenate containing certified levels of the PAHs evaluated in this procedure. Table 11 provides the acceptable range (defined as 70%-130% of certified value ± uncertainty). A representative chromatogram generated from the analysis of SRM 1974b is displayed in Figure 4. The results of this analysis, completed in triplicate, are summarized in Table 11. For the three preparations done in this study, at least eight of the PAHs determined were within the acceptable range. Note that the PMT gain on the FLD was set to 15 for

analysis of the SRM. Additionally, as described previously a 1:10 concentration of the extract was performed prior to analysis.

Table 11. PAH recoveries from NIST SRM 1974b.

Compound	Average Retention Time (n = 3) min	% RSD	Acceptable Range (mg/kg)	SRM Prep 1 Determined Amount (mg/kg)	SRM Prep 2 Determined Amount (mg/kg)	SRM Prep 3 Determined Amount (mg/kg)	Average Determined Amount (n = 3) (mg/kg)	% RSD
naphthalene	3.073	0.85%	1.6-3.3	5.98	11.77	8.82	8.86	32.7%
Fluorine	5.133	0.85%	0.3-0.7	**0.514**	**0.360**	**0.371**	0.415	20.7%
phenanthrene	5.814	0.54%	1.7-3.5	**2.98**	**2.27**	**1.92**	2.39	22.6%
Anthracene	6.642	0.08%	0.3-0.8	0.144	0.276	0.082	0.167	59.2%
fluoranthene	7.324	0.08%	11.5-23.1	**13.9**	**16.8**	**15.2**	15.3	9.8%
Pyrene	7.862	0.08%	12.2-24.2	**17.58**	**21.75**	**17.77**	19.03	12.4%
benz[a]anthracene	9.551	0.14%	2.9-6.9	**3.63**	**4.56**	**4.01**	4.07	11.5%
chrysene	10.115	0.08%	3..7-9.5	**9.2**	11.0	**8.0**	9.4	16.1%
Benzo[b]fluoranthene	11.807	0.10%	4.1-9.2	**5.07**	**5.94**	**4.66**	5.22	12.6%
Benzo[k]fluoranthene	12.919	0.10%	2.1-4.3	**2.21**	**2.81**	**2.13**	2.38	15.6%
benzo[a]pyrene	13.707	0.10%	1.7-4.1	**1.8**	**2.4**	**1.9**	2.1	16.3%
dibenz[a,h]anthracene	15.218	1.51%	0.2-0.5	1.704	2.376	2.758	2.279	23.4%
Benzo[g,h,i] perylene	16.166	0.14%	2.0-4.5	**2.78**	**3.63**	**2.61**	3.00	18.2%
Indeno[1,2,3-cd] pyrene	17.412	0.24%	1.4-2.9	0.41	0.40	1.11	0.64	63.4%

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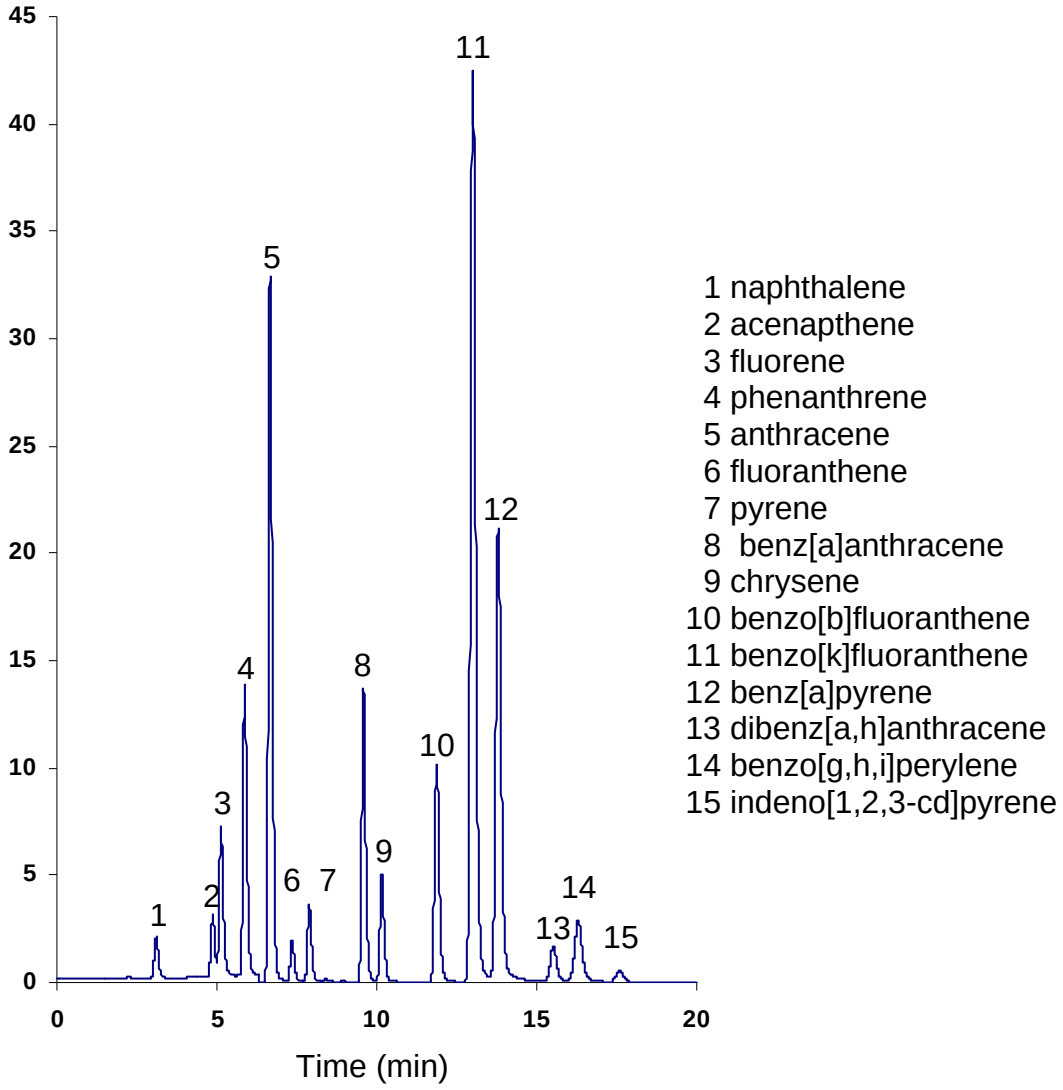

Figure 1. LC-FLD chromatogram of 15 PAH standard mix, 33 ng/mL each.

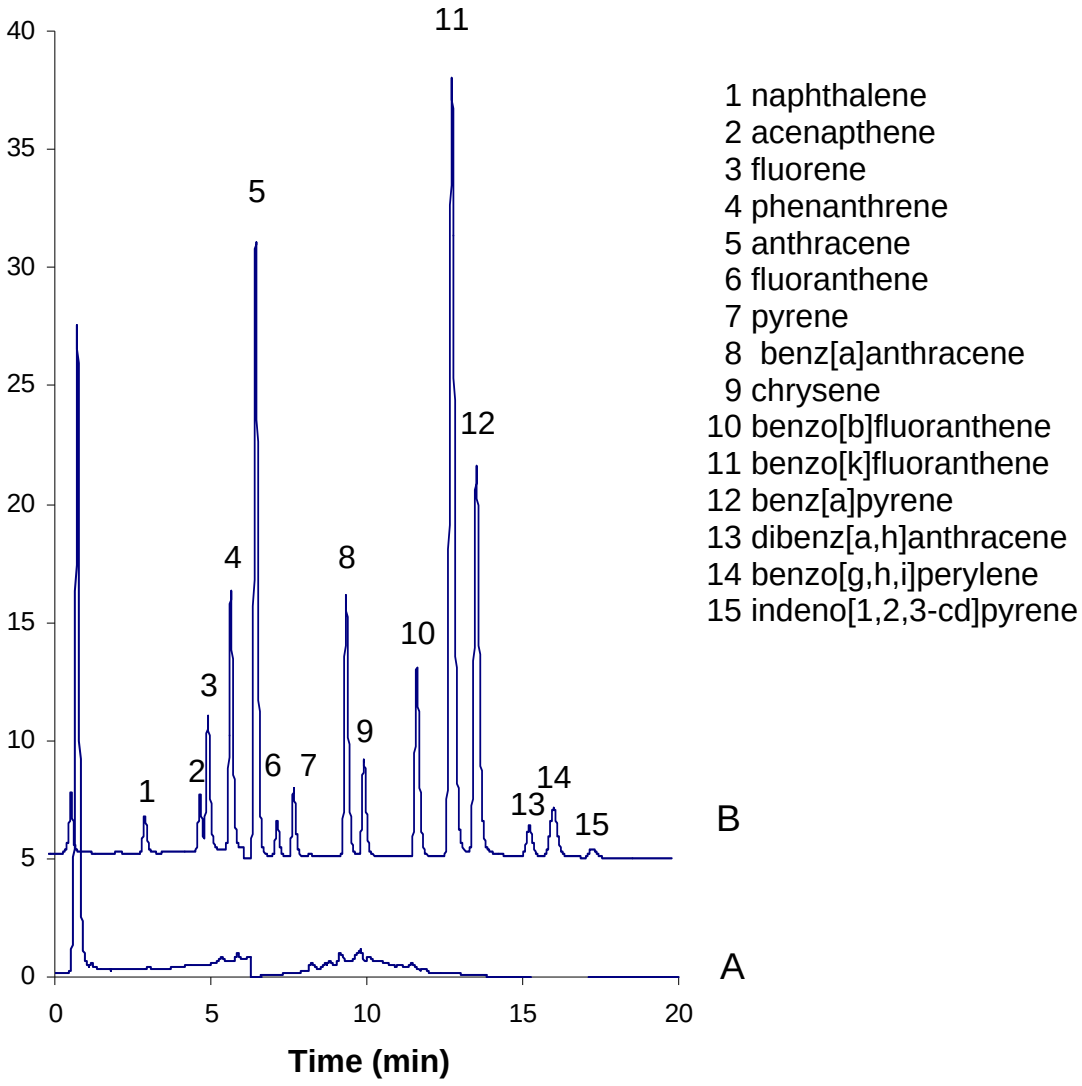

Figure 2. LC-FLD chromatograms obtained from oyster sample.
A) unfortified sample B) fortified with 15 PAH reference standard mixture at a level of 1 µg/g each (chromatogram offset by 5 luminescence units)

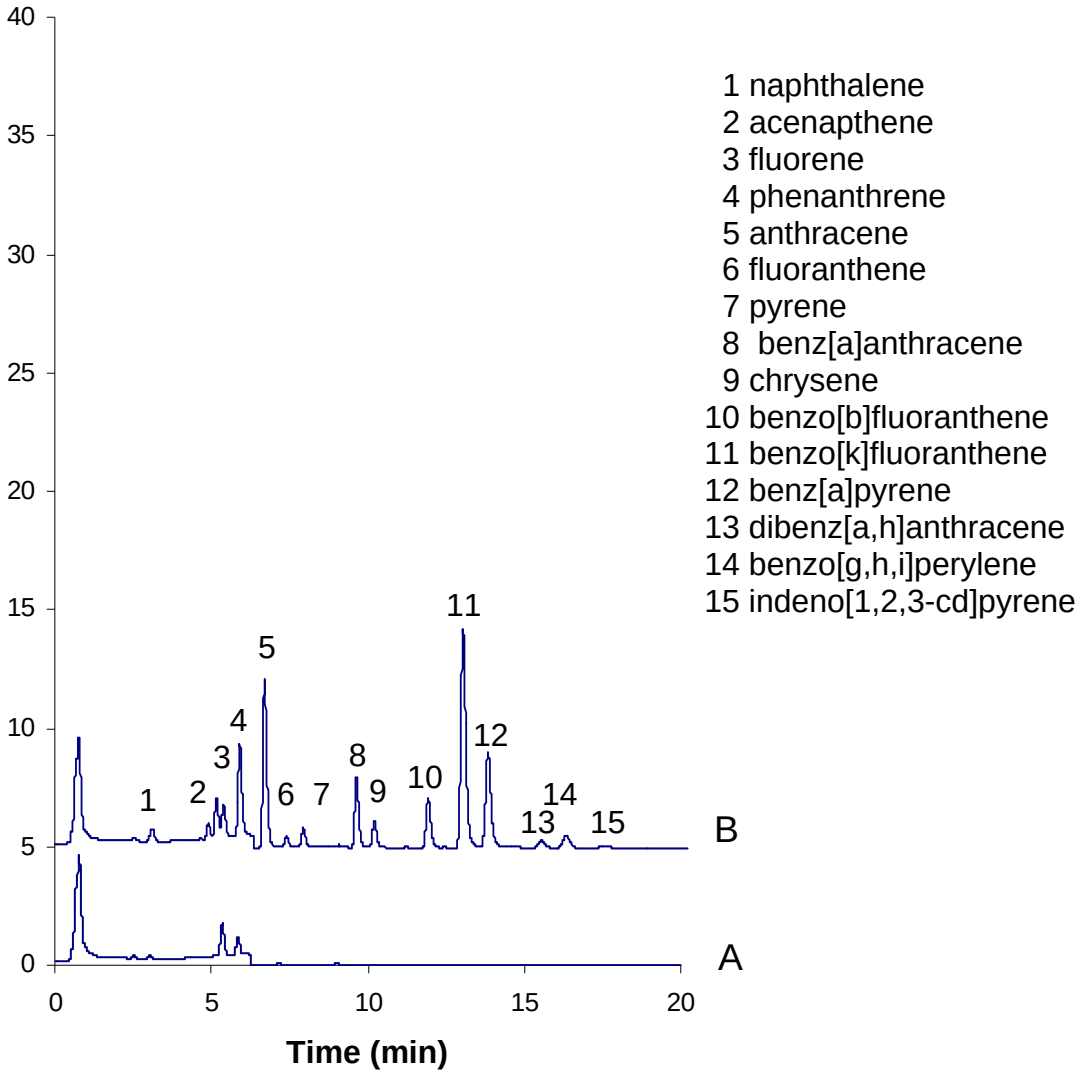

Figure 3. LC-FLD chromatograms obtained from finfish sample. A) unfortified sample B) fortified with 15 PAH reference standard mixture at a level of 0.025 µg/g each (chromatogram offset by 5 luminescence units)

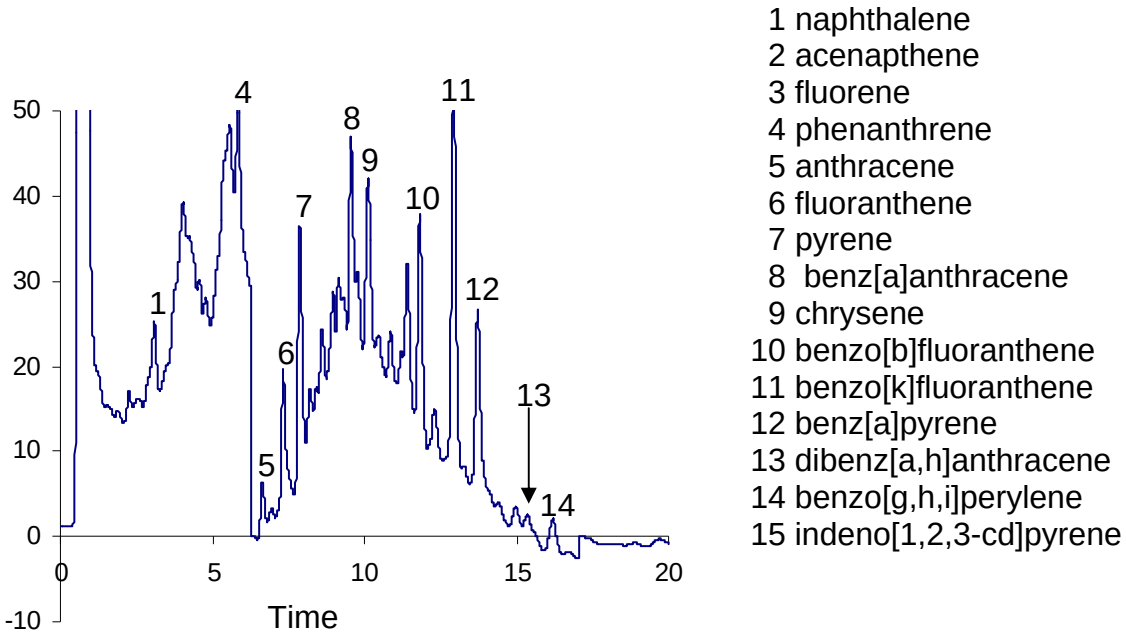

Figure 4. LC-FLD chromatogram obtained from NIST SRM 1974b, mussel tissue homogenate analyzed for 14 target PAHs.

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Appendix I – Preliminary Evaluation of Alkyl Homologues of PAHs

Samples of Oysters, finfish, shrimp and crab were fortified with a standard mixture containing 20 alkylated PAH homologues. Spike recovery data is provided below.

PAH Homologs - Oysters

Compound	l_{em}	RT check standard (min)	Spike Level (ng/g)	oyster spikes % recoveries sp1	sp2	sp3	average	%RSD
peak 1	352	4.145	50.0	130	129	127	**128**	1.4%
peak 2	352	4.491	50.0	127	119	120	**122**	3.6%
peak (s) 3	352	5.922	50.0	119	119	120	**119**	0.31%
peak (s) 4	352	7.193	25.0	105	108	107	**107**	1.7%
peak 5	352	8.048	NA	87	86	88	**87**	1.1%
Overall				111	113	112	**112**	0.59%
Averages				114	112	112	**113**	1.6%

peak 1 = biphenyl (front end), 1-methylnaphthalene
peak 2 = 2-methylnapthalene
peak (s) 3 = (multiple) dimethylnapthalenes, phenanthrene, 1,7 dimethylnapthalene
peak (s) 4 = 1-methyl phenanthrene (and multiple other methyl phenanthrenes?)
peak 5 = unknown

PAH Homologs – Shrimp

Compound	l_{em}	RT check standard (min)	Spike Level (ng/g)	shrimp spikes % recoveries sp1	sp2	sp3	average	%RSD
peak 1	352	4.145	50.0	108	110	103	**107**	3.5%
peak 2	352	4.491	50.0	102	103	103	**103**	0.36%
peak (s) 3	352	5.922	50.0	108	109	110	**109**	1.0%
peak (s) 4	352	7.193	25.0	114	114	115	**114**	0.51%
peak 5	352	8.048	NA	125	127	129	**127**	1.5%
Overall				111	112	113	**112**	0.71%
Averages				111	113	112	**112**	1.4%

peak 1 = biphenyl (front end), 1-methylnaphthalene
peak 2 = 2-methylnapthalene
peak (s) 3 = (multiple) dimethylnapthalenes, phenanthrene, 1,7 dimethylnapthalene
peak (s) 4 = 1-methyl phenanthrene (and multiple other methyl phenanthrenes?)
peak 5 = unknown

PAH Homologs -
Crab

Compound	l_{em}	RT check standard (min)	Spike Level (ng/g)	crab spikes % recoveries sp1	sp2	sp3	average	%RSD
peak 1	352	4.145	50.0	118	126	115	**120**	4.7%
peak 2	352	4.491	50.0	110	114	97	**107**	8.19%
peak (s) 3	352	5.922	50.0	111	111	102	**108**	4.9%
peak (s) 4	352	7.193	25.0	118	114	114	**115**	2.16%
peak 5	352	8.048	NA	120	118	118	**119**	0.8%
Overall				115	113	108	**112**	3.09%
Averages				115	117	109	**114**	4.1%

peak 1 = biphenyl (front end), 1-methylnaphthalene
peak 2 = 2-methylnapthalene
peak (s) 3 = (multiple) dimethylnapthalenes, phenanthrene, 1,7 dimethylnapthalene
peak (s) 4 = 1-methyl phenanthrene (and multiple other methyl phenanthrenes?)
peak 5 = unknown

PAH Homologs -
Fish

Compound	l_{em}	RT check standard (min)	Spike Level (ng/g)	fish spikes % recoveries sp1	sp2	sp3	average	%RSD
peak 1	352	4.145	50.0	119	121	118	**119**	1.2%
peak 2	352	4.491	50.0	119	118	111	**116**	3.9%
peak (s) 3	352	5.922	50.0	114	118	114	**115**	2.1%
peak (s) 4	352	7.193	25.0	115	120	115	**117**	2.0%
peak 5	352	8.048	NA	122	122	122	**122**	0.10%
Overall				115	119	115	**117**	1.9%
Averages				118	120	116	**118**	1.9%

peak 1 = biphenyl (front end), 1-methylnaphthalene
peak 2 = 2-methylnapthalene
peak (s) 3 = (multiple) dimethylnapthalenes, phenanthrene, 1,7 dimethylnapthalene
peak (s) 4 = 1-methyl phenanthrene (and multiple other methyl phenanthrenes)
peak 5 = unknown

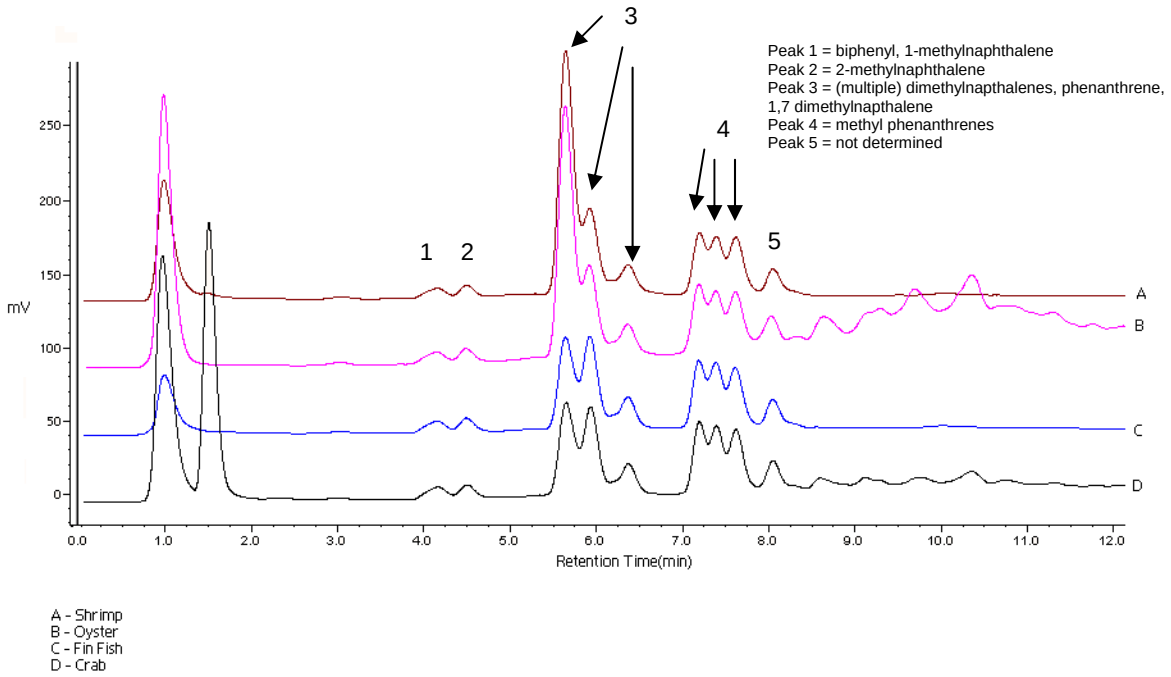

Figure A. Overlay of LC-FLD chromatogram obtained from shrimp, oyster, finfish and crab fortified with standard mixture containing 20 PAH alkyl homologues.

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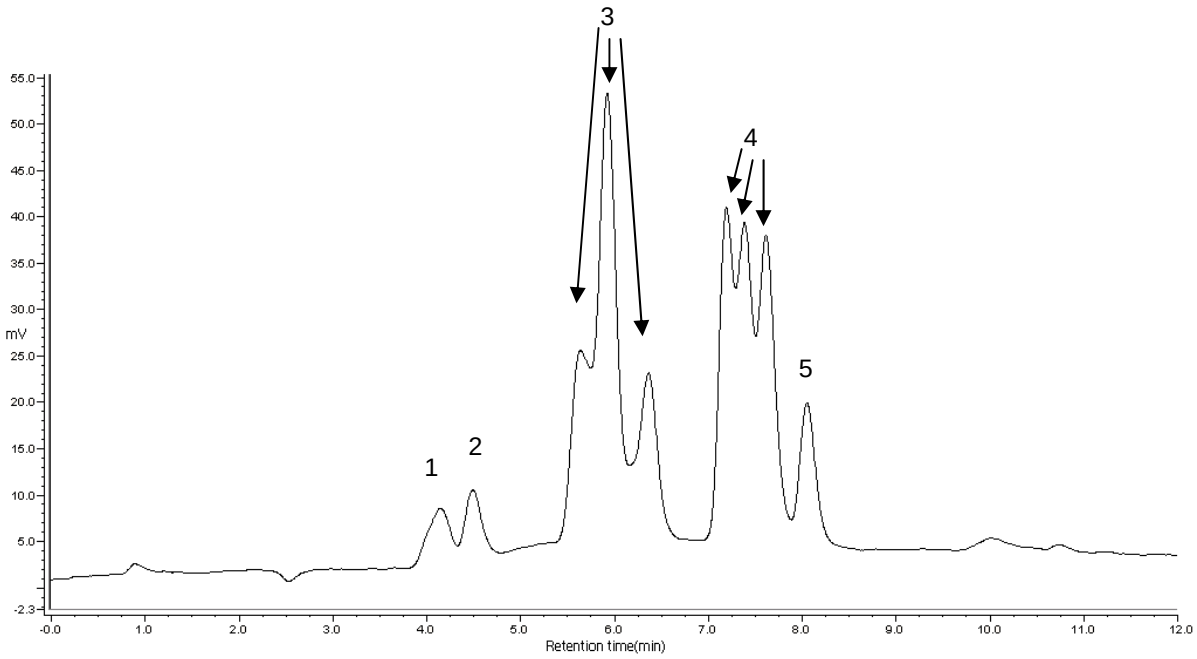

Figure B. LC-FLD chromatogram obtained standard mixture containing 20 PAH alkyl homologues.